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Identification of Genes Involved in *Neisseria meningitidis* Colonization

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Neisseria meningitidis **is a worldwide cause of meningitis and septicemia leading at least to 50,000 deaths every year. Nevertheless, *N. meningitidis* is also a commensal bacterium that asymptomatically colonizes the epithelial cells of the nasopharynx of 10 to 30% of healthy individuals. Occasionally, *N. meningitidis* crosses the nasopharyngeal barrier and enters the bloodstream. During bacteremia, *N. meningitidis* may adhere to endothelial cells of brain vessels and invade meninges. To identify the genes required for meningococcal host colonization, we screened a signature-tagged transposon mutagenesis library using an innovative *in vitro* colonization model in order to identify mutants displaying decreased capacity to colonize human epithelial cells. Approximately 1,600 defined insertion mutants of invasive serogroup C strain NEM8013 were screened. Candidate mutants were tested individually for quantification of bacterial biomass with confocal microscope and COMSTAT software. Five mutants were demonstrated to exhibit significantly decreased colonization ability. The identified genes, including *narP* and *estD*, appeared to be involved in adaptation to hypoxic conditions and stress resistance. Interestingly, the genes *fadD1*, *nnrS*, and *NMV_2034* (encoding a putative thioredoxin), prior to this study, had not been shown to be involved in colonization. Therefore, we provide here insights into the meningococcal functions necessary for the bacterium to adapt to growth on host cells.**

Neisseria meningitidis is a human pathogen that colonizes exclusively the nasopharynges of 10 to 30% of the human population (1). The ability of the bacterium to grow on the surfaces of epithelial nasopharyngeal cells is therefore crucial for its survival as a commensal organism. Disease occurs when an invasive strain of *N. meningitidis* crosses the nasopharyngeal epithelium and enters the bloodstream. Once in the blood vessels, bacteria are able to survive and multiply. Indeed, meningococcus is protected from the killing action of complement mainly by its polysaccharidic capsule but also by a sialylated lipooligosaccharide and by factor H-binding protein (2). During bacteremia, *N. meningitidis* may adhere to the endothelial cells of the brain microvessels, invade the meninges, and multiply in the cerebrospinal fluid. Since humans are the only known reservoir of *N. meningitidis*, its ability to efficiently colonize pharyngeal epithelial cells is crucial for its survival and transmission but is also a prerequisite for invasive meningococcal disease. Paradoxically, although deciphering the nasopharyngeal colonization process constitutes a prerequisite to understand how the transition from commensalism to pathogenicity occurs, the exact conditions allowing *N. meningitidis* to cross the epithelial barrier are still not known.

Initial, early interaction of *N. meningitidis* with epithelial cell surface has been studied extensively using *in vitro* cell culture and more recently using tonsil or nasal tissue (3, 4). These studies demonstrated that upon contact with human cells, the meningococcus initially adheres as microcolonies using filamentous organelles named type IV pili (T4P) (5). In a second step of infection, bacteria form a layer tightly attached to host cell surface (6, 7). Then, multiplication of bacteria leads to the formation of a biofilm structure several tens of microns thick (8). Biofilms are defined as surface-attached microbial communities embedded in a self-produced matrix. The ability of the meningococcus to form biofilm has been demonstrated both *in vivo* and *in vitro* (9). Invasive strains of meningococcus are systematically encapsulated, which is necessary for their survival in the bloodstream (10). Nevertheless, it is known that capsular polysaccharide confers a negative charge and hydrophilicity to the encapsulated organism. The first reports on meningococcal biofilm initially reported that presence of the capsule, which is a constant trait of invasive strains of meningococcus, inhibited biofilm formation by *N. meningitidis* over inert substratum such as plastic, polystyrene and glass, under static or flow conditions while unencapsulated strains were able to form biofilms on abiotic surfaces (11, 12). More recently, it has been evidenced that wild-type (WT) encapsulated *N. meningitidis* was able to form biofilms on human tissue culture cells in a flow chamber (8). In addition, proteomic and transcriptomic studies comparing planktonic versus biofilm grown meningococcus or gonococcus over inert surface have highlighted the role of enzymes involved in denitrification and adaptation to reactive oxygen species (13–15).

Signature-tagged transposon mutagenesis (STM) screening has been widely used to identify genes involved in colonization and biofilm formation in many human pathogens. In the present study, we screened an STM library using an original *in vitro* model of colonization in order to identify genes involved in colonization under constant flow shear stress. Five mutants were identified that were inactivated for *estD*, *narP*, *nnrS*, *fadD1*, and *NMV_2034*. The corresponding disrupted genes appeared to be mainly involved in adaptation to hypoxic conditions.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. *N. meningitidis* strain NEM8013 used in the present study is a piliated, Opa$^-$, Opc$^-$, PilC1$^+$, and PilC2$^+$ serogroup C clinical isolate (16).

Neisseria was grown at 37°C in moist atmosphere containing 5% CO_2 on GC medium base (GCB; Difco) containing Kellogg's supplement. *Escherichia coli* strain Top10 (Life Technologies) was used for DNA cloning and plasmid propagation. *E. coli* was grown on Luria-Bertani (LB) agar or in liquid medium. For antibiotic selection of *E. coli* strains, kanamycin was used at 30 μg ml^{-1}. To select strains derived from *N. meningitidis* strain 8013, the kanamycin concentration was 100 μg ml^{-1}, chloramphenicol was used at 5 μg ml^{-1}, and erythromycin was used at 2 μg ml^{-1}. STM mutants were cultured at 37°C on GC agar plates containing nutritional supplements and 100 μg of kanamycin/ml. *N. meningitidis* NEM8013 expressing a green fluorescent protein (GFP) was obtained by transformation of plasmid pAM239 (17). GFP-expressing mutants were obtained by transformation of genomic DNA of STM mutants into a WT GFP strain.

Epithelial cell culture conditions. The T84 human colonic carcinoma-derived epithelial cells and the pharynx carcinoma-derived FaDu epithelial cells were obtained from the American Type Culture Collection. Both cell lines were grown in Ham F-12 medium (PAA Laboratories) supplemented with 10% fetal calf serum (FCS; PAA Laboratories), 20 mM HEPES (PAA Laboratories), and 1% penicillin-streptomycin-amphotericin (PSA; PAA Laboratories). Cells were grown at 37°C in a humidified incubator under 5% CO_2. Medium without PSA is thereafter referred as infection medium (IM) and used to grow STM mutants.

***In vitro* screening of STM library for colonization defective mutants.** Laminar flow chamber experiments were performed on disposable flow chambers composed of six independent flow channels (μ-Slide VI 0.4 purchased from Ibidi, surface area = 0.6 cm^2 per channel) coated with 5 μg of rat tail collagen type I/cm^2. T84 or FaDu cells were seeded in the six channels at a density of 0.3×10^5/cm^2 and incubated for ~7 days at 37°C in 5% CO_2 until confluent. A microscopic examination of the cell layers was performed before each flow assay and only channels with a uniformly confluent layer were used. When pools of mutants were assayed, clones were resuspended individually in IM in a 96-well plate and incubated with shaking at 37°C. After 2 h of growth, the OD_{600} was measured on a microtiter plate reader, and an equivalent amount of each mutant strain was pooled. The suspension containing the pooled mutants was adjusted to an OD_{600} of 0.5 in IM and 60 μl was used to inoculate duplicate channels of a microslide containing epithelial cell monolayer. The remaining suspension is referred to as the input pool. Bacteria were allowed to adhere to epithelial cell monolayer for 1.5 h without flow. At 1.5 h postinfection, a continuous flow of IM containing 3 μg of vancomycin/ml was applied for 18 h at a constant flow rate of 0.04 ml/min using a syringe pump (Harvard Apparatus). The flow chamber was placed in an incubator thermostated at 37°C with 5% CO_2 throughout the experiment. After 18 h, the recovered bacteria, i.e., the bacteria obtained from channel aspiration and/or released after trypsin treatment, were plated on GCB agar plates. After an overnight incubation at 37°C, colonies constituting the output pool were scraped from plates and resuspended in nucleus lysis solution (Promega). Chromosomal DNA extraction was performed using Wizard Genomic DNA purification kit (Promega) on the input and output mutant pools.

Tag amplification. For the present study, we developed a screen method based on PCR. Since each mutant present in a pool is labeled with a unique tag, we designed 48 oligonucleotides complementary to the tag region to perform specific amplification of each tag (see Table S1 in the supplemental material). Chromosomal DNA isolated from input and output mutant pools were used as a template in PCR amplification with the 48 pairs of primers.

Confocal microscopy and image analysis of biofilms. The Ibidi μ-Slide flow chambers allow direct observation with inverse microscopy through its transparent plastic bottom. Prior to infection, epithelial cell monolayers grown in μ-Slide were stained with cytoplasmic Cell Tracker Orange CMTMR (Life Technologies) according to the manufacturer's instructions. The channels were then filled with a bacterial suspension of GFP-expressing strain, and the flow chamber was incubated 1.5 h to allow adhesion. After 1.5 h, a continuous flow of IM containing 3 μg of vancomycin/ml and 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) was applied for 18 h at 0.04 ml/min.

TABLE 1 Oligonucleotide primers used in this study

Primer	Sequence (5′–3′)
FcompNMV2034	CCTTAATTAAGGAGTAATTTTATGAAAAAGAAACTGCTTTCG
RcompNMV2034	CGACAGTACTTTAACCGAAAACATCCGC
FcompNMV845	CCTTAATTAAGGAGTAATTTTATGGAAAAAATCTGGTTAG
RcompNMV845	CGACAGTACTTTATTTCCCGGTACTTTGG
Ftag	TTTACTGGATGAATTGTTTTAGTACCT
Rtag	GCTGATAAGTCCCCGGTCTA
Fcst	GCATCAGGCTCTTTCACTCC

All microscopic observations and image acquisitions were performed on a Leica SP5 confocal microscope. Images were obtained using a ×40/1.3 Plan Apo oil objective lens. At the time of confocal acquisition, the cells were examined using red channel to assess the integrity of the monolayer. Three-dimensional biofilm structures reconstructions were generated by using the IMARIS software package (Bitplane AG). Biofilm development was quantified with the COMSTAT computer program using biomass and average thickness parameters (18). The results are expressed as a percentage of the biofilm produced by the wild-type strain, which is set to 100%. Values represent the means of three independent experiments, with the acquisition of at least four image stacks per each channel. The data were examined for significance using the Student *t* test.

Construction of complemented mutants. To complement the *NMV_2034* and *fadD1* mutants, the wild-type genes were amplified using chromosomal DNA of strain 8013 as a template and primers FcompNMV2034/RcompNMV2034 and FcompNMV845/RcompNMV845, respectively. Forward primers contained overhangs with restriction sites for PacI, whereas reverse primers contained overhangs with restriction sites for ScaI (Table 1). These PCR fragments were restricted with PacI and ScaI and cloned into PacI/ScaI-cut pGCC4 vector, adjacent to lacIOP regulatory sequences (21). This placed *NMV_2034* or *fadD1* under the transcriptional control of an IPTG-inducible promoter within a DNA fragment corresponding to an intragenic region of the gonococcal chromosome conserved in *N. meningitidis*. The *NMV_2034* or *fadD1* inducible allele was then introduced into the chromosome of *NMV_2034* or *fadD1* mutant by homologous recombination.

Susceptibility to H_2O_2 and PxB. The disc diffusion method (Kirby-Bauer testing) was used to test susceptibility to H_2O_2 and polymyxin B (PxB). Bacteria from the agar plate were resuspended in 5 ml of brain heart infusion broth to an optical density at 600 nm of 0.15 and spread with a cotton swab onto a GCB agar plate. Paper disks 6 mm in diameter were placed on the swabbed plate and saturated with 10 μl of test agent. After 18 h of incubation, the zones of growth inhibition were measured. The reagents tested were 3% H_2O_2 and PxB at a concentration of 300 μg/ml (both purchased from Sigma).

RESULTS

Establishment of an *in vitro* colonization model. In order to study the late stages of the interaction of epithelial cells with encapsulated meningococcus and to identify as-yet-unidentified meningococcal functions required for the colonization process, we developed an innovative *in vitro* colonization model (Fig. 1A). We took advantage of the Ibidi microslides VI, which contain six channels (flow chambers) and allow the parallel testing of six pools of mutants in a single experiment. These microslides have been previously used to study early adhesion events of meningococcus on endothelial cell monolayer under defined shear stress conditions that mimic blood flow (17). We used this miniaturized laminar flow chamber to ensure a permanent renewal of the

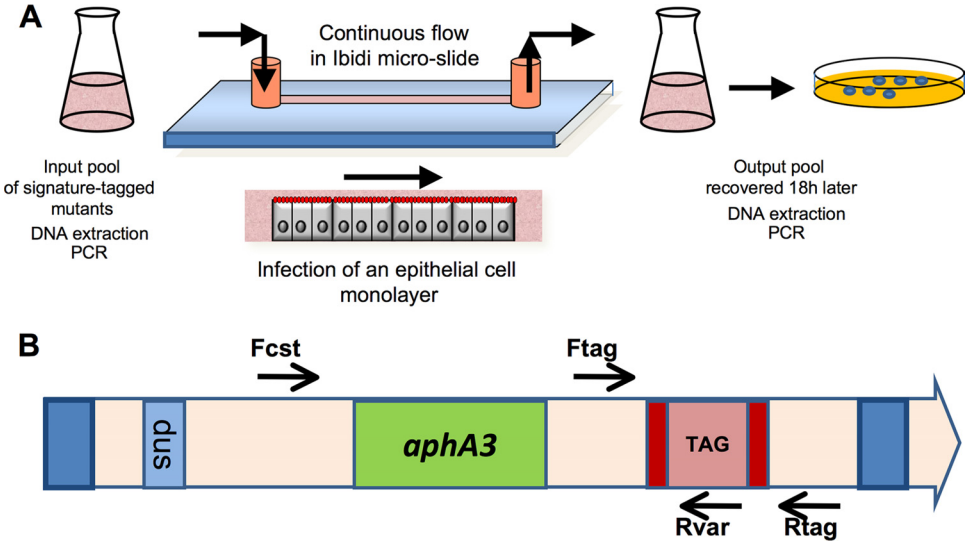

FIG 1 Schematic of signature-tagged mutant screen for identification of genes involved in epithelial cells colonization. (A) Pools of mutants (input pools) were used to infect an epithelial cell monolayer in an Ibidi microslide and recovered 18 h later (output pools). The presence of each mutant was investigated in both input and output pools by a specific PCR amplification of the tags. Mutants present in the input pool but absent in the output pool were further investigated. (B) Representation of the transposon found in STM mutants. Ftag and Rtag primers were used to sequence the 48 different tags. Fcst, along with 48 different oligonucleotides (Rvar), was used to screen the library, yielding a 500-bp PCR product when a mutant is present in a pool. DUS, meningococcal DNA uptake sequence; *aphA3*, kanamycin resistance gene.

growth medium. These growth conditions allowed the formation of a bacterial biofilm over the epithelial cell monolayer. It is the first time that such a model has been used to screen a mutant library in *Neisseria*.

Development of an optimized PCR-based screening method. Our previously described transposon library (10, 16) of 4,548 mutants is organized in 96 pools of 48 mutants labeled with 48 different tags. The transposon contains a variable tag of 40 bp and a constant region, including a kanamycin resistance cassette (Fig. 1B). Therefore, each mutant in a defined pool is labeled with a unique tag. For the present study, in order to simplify the detection of mutants present in both input and output pools, we developed a screening method based on PCR instead of the classical hybridization method previously used (19, 20). This screening method is easier and less time-consuming than hybridization procedures.

The 48 different tags were sequenced (using Ftag and Rtag primers; Table 1), and 48 oligonucleotides complementary to ~20 bp of the tag region were designed, along with a unique primer in the constant region of the transposon (Table 1 and see Table S1 in the supplemental material). PCR amplifications with the 48 pairs of primers yielded a 500-bp product. Sensitivity and specificity testing of the 48 pairs of oligonucleotides were performed by PCR to evaluate cross-amplification of the tags. When necessary, a new oligonucleotide was designed to improve sensitivity or specificity. Tags 5 and 25 were extremely similar and could not be differentiated by a specific PCR; therefore, mutants harboring these tags were never mixed in the same pool to perform infection.

Screening for mutants impaired in epithelial cell colonization. Among 4,548 insertion mutants in the capsulated *N. meningitidis* strain 8013, the location of 3,625 transposons has been mapped (16). Several genes had multiple insertions of the transposon leading to a redundancy in the library. In order to avoid testing numerous mutants in the same genes, we selected 976 unique transposon insertions in well-defined open reading frame and 639 transposon insertions located in intergenic regions.

Preliminary tests of our *in vitro* colonization model were initially conducted with pools, including a nonpiliated *pilD* mutant to verify the absence of this tagged mutant in the output pools and validate our novel model of colonization. As expected, the nonpiliated mutants were not recovered in the output pools. Next, the 1,615 mutants, grouped in 96 pools of ~17 mutants, were assayed in duplicate on T84 epithelial cells monolayers grown in Ibidi microslides VI 0.4. We identified 312 potentially attenuated candidates from this first round of screening that were absent in output pools recovered at 18 h postinfection. Phase variation which results in the variability of the surface antigens could generate variants with a colonization defect unlinked to the transposon insertion. To rule out this possibility, we transformed into the parental strain each of the 312 mutations. We assembled new pools of mutants and tested them a second time in the *in vitro* colonization model. In this second-round of screening, we identified 85 candidates. We determined the individual growth curves and discarded mutants displaying altered growth kinetics compared to the wild-type strain in liquid cultures. Several nonpiliated mutants (i.e., *pilD*, *pilG*, *pilJ*, *pilH*, and *pilM* mutants) were found during the screening but were not investigated further since mutants with an initial adhesion defect were excluded from the study.

Quantification of the biomass produced by candidate mutants. We then performed individual testing of the 55 remaining mutants using confocal microscopy to evaluate the formation of the biomass. Each mutation was transformed into a strain expressing the GFP under the control of an IPTG-inducible promoter encoded by pAM239 plasmid (17). Using a confocal microscope and COMSTAT software, we compared the biomasses of mutants to that of the parental GFP strain. To avoid the phase variation effect, the experiment was repeated with three independent clones. We found five mutants (i.e., the *estD*, *narP*, *nnrS*, *fadD1*,

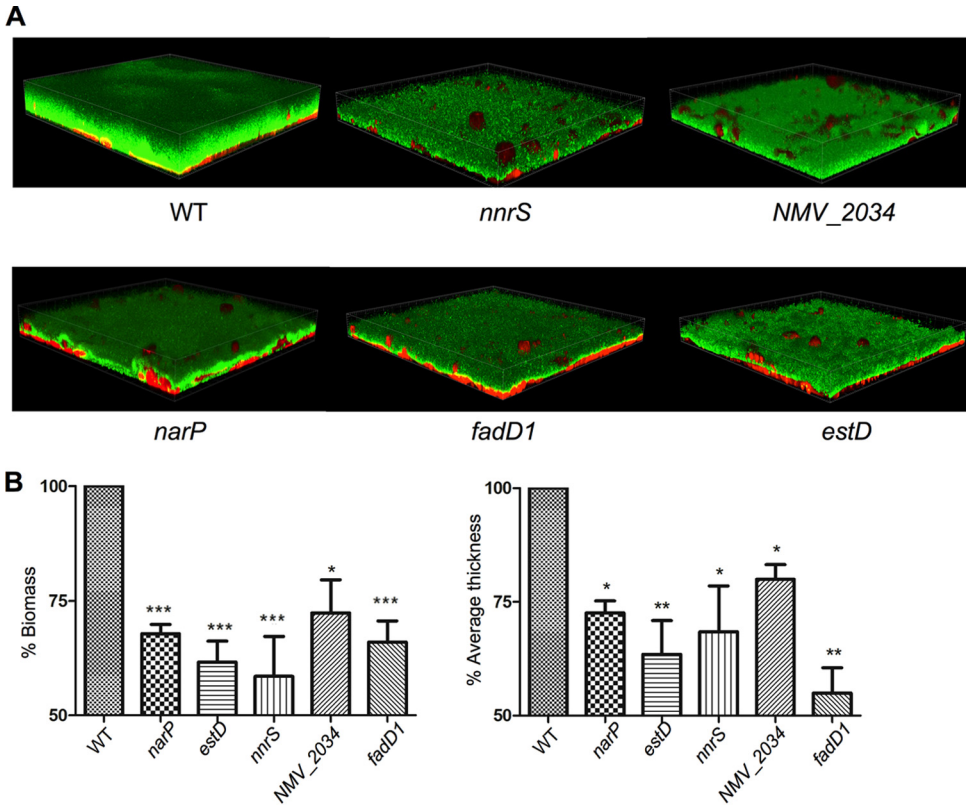

FIG 2 Meningococcal biofilm formation over epithelial cells. (A) Three-dimensional biofilm structures reconstructed with Imaris software of wild-type and five mutant strains grown over T84 epithelial cells for 18 h. Representative images of experiments performed on at least three different occasions are shown. (B) Analyses of biomass and average thickness using COMSTAT software of wild-type and five mutant strains identified in STM screening. During the screening process, wild-type and mutant strains were grown over T84 epithelial cells for 18 h. At least three independent experiments were performed. The results are normalized as a percentage of the mean biomass of the wild-type strain, which was set to 100%. Error bars indicate the standard errors of the mean (SEM). ***, $P < 0.005$; **, $P < 0.01$; *, $P < 0.05$ (Student *t* test).

and *NMV_2034* mutants) with a reproducible biomass defect compared to the parental strain (Fig. 2 and Table 2). Biofilms formed by the five mutants were also significantly reduced in average thickness compared to wild-type biofilms (Fig. 2). None of the mutants exhibited initial adhesion defect to human epithelial cells at 3 h postinfection (data not shown). The biomass defect of the five mutants was also observed when mutants were grown on FaDu epithelial cell line instead of T84 cell line (data not shown). In order to verify the absence of polar effect and to ensure that the phenotype was attributable to transposon insertion, *NMV_2034* and *fadD1* mutants were complemented in *trans* with a wild-type copy of the gene using pGCC4 (21). Complementation reversed the colonization defect observed in both mutant strains (Fig. 3 and 4).

Genes identified in our *in vitro* colonization model. Our screen identified *estD*, which encodes esterase D. *narP* encodes the NarP regulator of the two-component system NarP/NarQ, which is involved in the denitrification process. *nnrS* encodes a heme- and copper-containing membrane protein (22) that plays a role in the metabolism of nitrogen oxides. *NMV_2034* encodes a putative membrane-associated thioredoxin. The *fadD1* gene encodes FadD1, a long-chain fatty acyl-coenzyme A (CoA) ligase that activates long-chain fatty acids by acyl-CoA ligation.

Since cationic antimicrobial peptides (CAMPs) are important in innate host defense system and are released by host cells, including epithelial cells, we examined the effect of CAMPs on our five mutants using polymyxin B (PxB), which is a cyclic lipopeptide frequently used to test CAMPs susceptibility. Despite the fact that

TABLE 2 Genes identified using our *in vitro* colonization model

Locus tag				
NEM8013	Z2491	MC58	Gene	Predicted function
NMV_1091	*NMA1519*	*NMB1305*	*estD*	Putative esterase D
NMV_1178	*NMA1419*	*NMB1250*	*narP*	Putative two-component system transcriptional regulator protein
NMV_2034	*NMA0612*	*NMB1845*		Putative membrane-associated thioredoxin
NMV_0482	*NMA2046*	*NMB0439*	*nnrS*	Putative NnrS-like protein (nitrite/nitric oxide reductase-related protein)
NMV_0845	*NMA1743*	*NMB1555*	*fadD1*	Long-chain fatty acid-CoA ligase

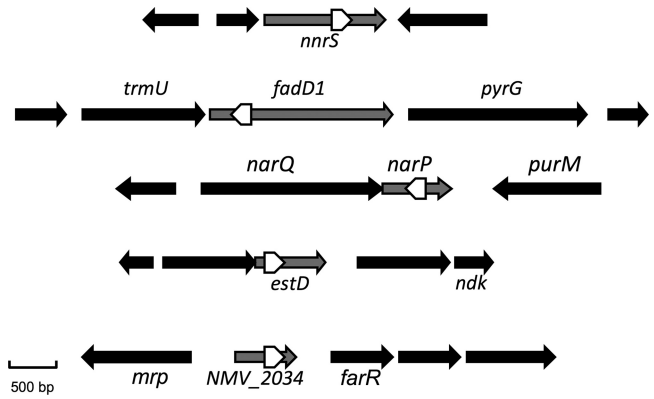

FIG 3 Gene organization around the five genes identified by STM. Gray arrow, gene identified; unlabeled black arrows, genes of unknown or putative function; white arrows, transposon insertion and orientation (transposon sizes are not to scale). Designations: *trmU*, tRNA-methyltransferase; *pyrG*, CTP synthase; *narQ*, nitrate/nitrite sensor protein; *purM*, phosphoribosylaminoimidazole synthetase; *ndk*, nucleoside diphosphate kinase; *mrp*, multidrug resistance-associated protein; *farR*, regulator of fatty acid efflux pump.

N. meningitidis is intrinsically resistant to PxB, mutants displaying increased sensitivity to PxB have been described (23). In addition, a decrease in resistance to oxidative stress is also known to be linked with biofilm defect and could explain the observed colonization defect (24). Therefore, we also examined the susceptibility of our five mutants to oxidative stress (H_2O_2). However, all five mutants exhibited the same level of resistance in Kirby-Bauer testing with H_2O_2 and PxB compared to parental strain.

DISCUSSION

We assayed an ordered library of transposon-generated mutants of *N. meningitidis* strain 8013 for isolates that showed impaired ability to colonize an epithelial cell monolayer compared to the WT parental strain. In a screen of a subset of ~1,600 mutants representative of this mutant library, we have identified five mutants. Except *estD* and *narP* that are known to be involved directly (*estD*) or indirectly (*narP*) in biofilm formation (25–27), the three other genes have never been linked to biofilm formation.

Since meningococcus is restricted to human host, no animal model is available for studying the meningococcal colonization process. Several other models have been used, including human organ culture model of adenoid or turbinate tissue (3, 4). One of the major problems encountered with tissues is an important host-to-host variation (28). A recent study was conducted with explants of tissue from inferior turbinates resected from patients (29). The ability to colonize nasopharyngeal tissue was assayed for 576 mutants and allowed the identification of eight defective mutants including two nonpiliated mutants and three mutants in genes encoding proteins of unknown function. The fact that neither the screen performed by Exley et al. nor our own present study used a saturated mutant library in two different *in vitro* colonization models could explain why neither study identified the same loci. Moreover, we used a flow chamber experiment allowing the formation of a biofilm of several tens of microns in height. Thus, our system led to the identification of mutants with a defect in biofilm biomass accumulation (Fig. 4).

Several recent studies have attempted to identify genes involved in the colonization of either gonococcus or meningococcus by transcriptomic or proteomic comparison of biofilm versus planktonic growth. All of these studies have been conducted on inert substratum under a continuous flow of medium (13–15, 30). In gonococcus, genes found to be highly upregulated in biofilms included nitrite reductase gene (*aniA*) and nitric oxide reductase gene (*norB*), which are both part of the NarP regulon (15). Furthermore, *aniA* and *norB* gonococcus mutants were defective for biofilm formation on glass and cervical epithelial cells. The increased level of AniA expression in gonococcal biofilm was confirmed by a proteomic approach (14). A proteomic analysis of meningococcal biofilms (13) also reported changes in protein expression related to oxygen limitation and defense against reactive oxygen species.

We demonstrated that a *narP* mutant exhibited a biomass defect over epithelial cells. NarP is the regulator of the two-component system NarQ/NarP. This system has been mainly studied in the gonococcus and is involved in the denitrification process which comes into play when a bacterium is in an environment where oxygen is scarce or absent. A *narP* mutant in the gonococcus is severely attenuated in its ability to grow in a low-oxygen environment (31). The use of the denitrification pathway by *N.*

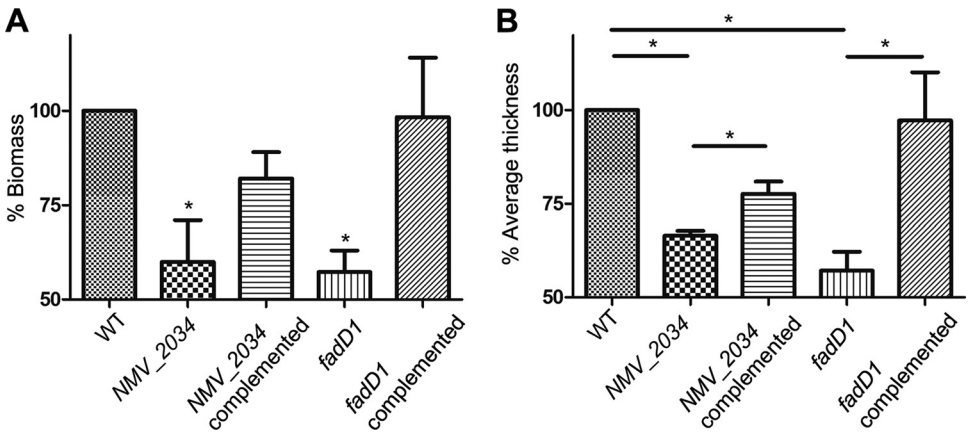

FIG 4 Analyses of the percent biomass (A) and the percent average thickness (B) using COMSTAT software of the WT and *NMV_2034* and *fadD1* mutants and their respective complemented strains. At least three independent experiments were performed. The results are normalized as a percentage of the mean biomass or average thickness of the wild-type strain, which is set to 100%. Error bars indicate the SEM. *, $P < 0.05$.

meningitidis in the nasopharynx is probably essential because this biological niche is colonized with a polymicrobial biofilm where oxygen cannot penetrate easily.

nnrS is a member of the *nnrR* regulon in *Rhodobacter sphaeroides* (32), which include genes required for the reduction of nitrite to nitrous oxide. NnrS is a heme- and copper-containing membrane protein (22). NnrS may have a role in the metabolism of nitrogen oxides (32). A study in *Vibrio cholerae* demonstrated that the expression of *nnrS* is controlled by NorR and that *nnrS* plays a role in NO resistance (33).

Several studies have shown that genes involved in defense against oxidative stress were required for biofilm formation. These genes include *oxyR*, *prx*, *gor* (24), and *trxB* (34) in gonococcus and *mntC* (13, 35) in meningococcus and gonococcus. Thioredoxins are oxidoreductase enzymes that play a role in reactive oxygen species response. They are maintained in a reduced state by thioredoxin reductase. The thioredoxin reductase TrxB participates in reactive oxygen and nitrogen species defense. A *N. gonorrhoeae trxB* mutant is defective in biofilm formation on glass and interestingly is more attenuated when grown over an epithelial cells monolayer instead of glass (34). In the present study, we identified NMV_2034, which is annotated as a putative membrane-associated thioredoxin. Nevertheless, no peptide signal could be predicted using SignalP or LipoP servers in strain 8013 sequence of *NMV_2034*. Since our *NMV_2034* mutant does not display increased susceptibility to hydrogen peroxide, further investigations are needed to understand the function of this putative thioredoxin.

Our screen also identified an *estD* mutant. This finding corroborates the results of Potter et al. (26), who previously investigated an *estD* mutant in the gonococcus. The authors showed that an *estD* mutant is impaired in biofilm formation when grown over epithelial cells in a flow chamber. The *estD* gene of the gonococcus strain (NGO0600) shares >92% nucleotide sequence identity with its meningococcal counterpart and is localized in a chromosomal region organized similarly to the meningococcus. Serine thioesterase EstD, together with alcohol dehydrogenase AdhC, is known to detoxify formaldehyde in meningococcus (36). In the gonococcus, the *estD* mutant exhibited a biofilm defect only when grown on an epithelial cell surface but not when grown on a glass surface. Furthermore, a meningococcal *estD* mutant grown over glass forms a biofilm mainly composed of nonviable bacteria. The observation that an *estD* mutant biomass defect was observed only over a living substratum sustained the hypothesis of an exogenous source of formaldehydes arising from epithelial cells. The reduced viability of the biofilm observed over glass with meningococcus is also consistent with an additional endogenous source of formaldehydes that accumulate in the *estD* mutant, leading to its premature death.

fadD gene encodes FadD, a long-chain fatty acyl-CoA ligase that activates long-chain fatty acids (LCFAs) by acyl-CoA ligation and also plays a role in the import of exogenous LCFAs (37). Several species such as *Pseudomonas aeruginosa*, *Mycobacterium tuberculosis*, or *N. meningitidis* possess multiple *fadD* homologs with potential differences in substrate preference. The *V. cholerae fadD* mutant was attenuated in an *in vivo* infant mouse cholera model due to downregulation of the major virulence genes in this mutant (38). Inactivation of *fadD* affects the virulence of several other bacterial pathogens, including the plant pathogen *Sinorhizobium meliloti* (39), *Salmonella enterica* serovar Typhimurium (40), and *P. aeruginosa* (41). Transcriptome or proteome profiling of a meningococcal *fadD* mutant could help to explain the colonization defect observed with this mutant.

In summary, we identified several *N. meningitidis* loci that contribute to the colonization of epithelial cells, which is a prerequisite step for invasive disease. Further characterization of mutants is necessary in order to understand the relationship between these different loci and meningococcal colonization. Due to the lack of animal model, *in vitro* biofilms grown in flow chambers are increasingly used to model meningococcal carriage. Our results demonstrate that STM combined with continuous-flow system for biofilm growth over epithelial cells is a powerful method for the identification of genes required for the colonization/biofilm formation process.

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